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Current Opinion in Biotechnology

DOI:
10.1016/j.copbio.2021.09.015

Published: 02/02/2022

Peer reviewed version

Calm and Frenzy: marine obligate hydrocarbonoclastic bacteria sustain ocean wellness.

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Abstract

According to current estimates, the annual volume of crude oil entering the ocean due to both anthropogenic activities and naturally-occurring seepages reaches approximately 8.3 million metric tons. Large discharges from accidents such as with tankers '*Amoco Cadiz'* and *M/T 'Haven'* and with the oil rig '*Deepwater Horizon'* have caused large-scale environmental disasters with extensive damage to the marine ecosystem. Typically, oil cleaning operations take months (and sometimes years) to bring the areas around an accident back to normality. The natural clean-up of petroleum spills in marine environments is carried out primarily by naturally-occurring, taxonomically diverse, obligate hydrocarbonoclastic bacteria (OHCB) that are capable of using hydrocarbons as a sole source of carbon and energy. The natural hosts of OHCB include a range of marine primary producers, e.g. unicellular photosynthetic eukaryotes and cyanobacteria, which have been documented as both, suppliers of hydrocarbon-like compounds that fuel the 'cryptic' hydrocarbon cycle (estimated to 100-500-fold more hydrocarbon than technogenic sources) and as a source of isolation of new OHCB. A very new body of evidence suggests that OHCB are not only the active early-stage colonizers of plastics and hence an important component of the ocean's 'plastisphere' but also encode an array of enzymes experimentally proven to act on petrochemical and bio-based polymers.

Introduction and updated taxonomical overview on marine OHCB

Some 15 years ago we reported on an ecophysiologically unusual group of marine microorganisms, termed 'obligate hydrocarbonoclastic (oil-degrading) bacteria (OHCB) [1••]. One of their most distinct metabolic peculiarities is their ability for utilizing almost exclusively hydrocarbons as a sole source of energy and carbon (a metabolic trait, called the "OHCB paradigm" [1••]). Here, we need to note that genetic loci of this physiological trait

are relatively easily transmissible between bacteria, which is known for decades e.g. for alkane hydroxylases/monooxygenases and P450 (CYP153) cytochromes (https://pubmed.ncbi.nlm.nih.gov/11390693/) or for a half-century, aromatic monooxygenases (TOL papers https://pubmed.ncbi.nlm.nih.gov/4418209/). Importantly, the phylogenies of these enzymes are uncoupled with the taxonomic placement of producing organisms (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4021335/) therefore the term OHCB cannot be strictly attributed to a particular taxon.

The ecological importance of OHCB in the biological removal of petroleum hydrocarbons from various polluted marine environments around the world has currently been evidenced during last dramatic oil spill disasters, such as aforementioned blowout of the Deepwater Horizon (DWH) oil rig (see references below). Since the discovery of the first obligate marine hydrocarbonoclastic bacteria being as only members of the class *Gammaproteobacteria*, their taxonomic diversity has not much been changed at the level of higher taxa. To date, of the eleven recognized genera, homing the OHCB species, only the genus *Planomicrobium* includes the species *P. alkanoclasticus* [2], order *Firmicutes* within the class *Bacilli,* has been added to the list. The rest of the well-established and novel OHCBs are the members of the class *Gammaproteobacteria* and are subdivided into four orders*: Cellvibrionales* (*Porticoccus* [3•]); *Nevskiales* (*Algiphilus* [4•] and *Polycyclovorans* [5•]); *Oceanospirillales* (*Alcanivorax* [6]*, Neptunomonas* [7]*, Oleibacter* [8]*, Oleiphilus* [9]*, Oleispira* [10] and *Thalassolituus* [11]) and *Thiothrichales* (*Cycloclasticus* [12]).

Initially characterized as highly specialized hydrocarbonoclastic bacteria that degrade either aliphatic (*Alcanivorax, Oleibacter, Oleiphilus, Oleispira, Thalassolituus*) or aromatic (*Cycloclasticus, Neptunomonas*) hydrocarbons [1••,13-15], OHCB possess a higher metabolic versatility than previously assumed. For example, in addition to the type species of the genus, *A. borkumensis* SK2^{T}, the genus *Alcanivorax*, currently includes 14 further

species with validly published names (https://lpsn.dsmz.de/genus/alcanivorax), some of which exhibit genome sizes much larger than that in the strain SK2^T and, in turn, utilize a larger range of growth substrates. More specifically, some *Alcanivorax* strains were found to be able to degrade simple sugars such as arabinose and glucose [16] and simple aromatic compounds, such as benzene, chlorobenzene, toluene [17] expanding the metabolic capability of this group of organisms, historically known as narrow specialists in degradation of aliphatic, branched hydrocarbons (including isoprenoids) and cycloalkanes [1••,6]. In concordance, some mussel and sponge symbionts isolated from deep-sea gas and oil seeps [18] were classified as the members of the genus *Cycloclasticus*, the genus of well-established primarily degraders of (poly)aromatic hydrocarbons in marine environments. They were found to be similar to free-living *Cycloclasticus* that bloomed during the DWH oil spill [19-24 and references therein]. Both these groups of bacteria were capable of degradation of short-chain alkanes ethane, propane and butane [18,19], thus highlighting the expanded role of these keystone species in the degradation of hydrocarbons released into marine environment. Of special attention are the recently uncovered novel OHCBs (*Algiphilus aromaticivorans*, *Polycyclovorans algicola*, *Porticoccus hydrocarbonoclasticus*) that represent novel genera and species and that were isolated from eukaryotic phytoplankton [3•,4•,5•]. All these isolates can use wide range of hydrocarbons, from aliphatic (C_{10} – C_{16}) and branched (phytane, pristane) to mono- (benzene, toluene, p-xylene, biphenyl) and polyaromatic hydrocarbons (naphthalene, anthracene, phenanthrene, pyrene, fluorene) as sole carbon sources for growth [14,15].

Marine OHCBs and the phycosphere

Marine OHCBs appear to be confined to the marine environment, where they tend to be highly enriched in petroleum-contaminated areas. However, in addition to polluted sites,

these organisms have been found in shallow, coastal and deep pristine areas around the world including polar regions, where no apparent hydrocarbon pollution has ever been recorded (Figure 1).

<<<<<<<<<<Figure 1 has to be inserted here >>>>>>>>>>>>>>>>>>

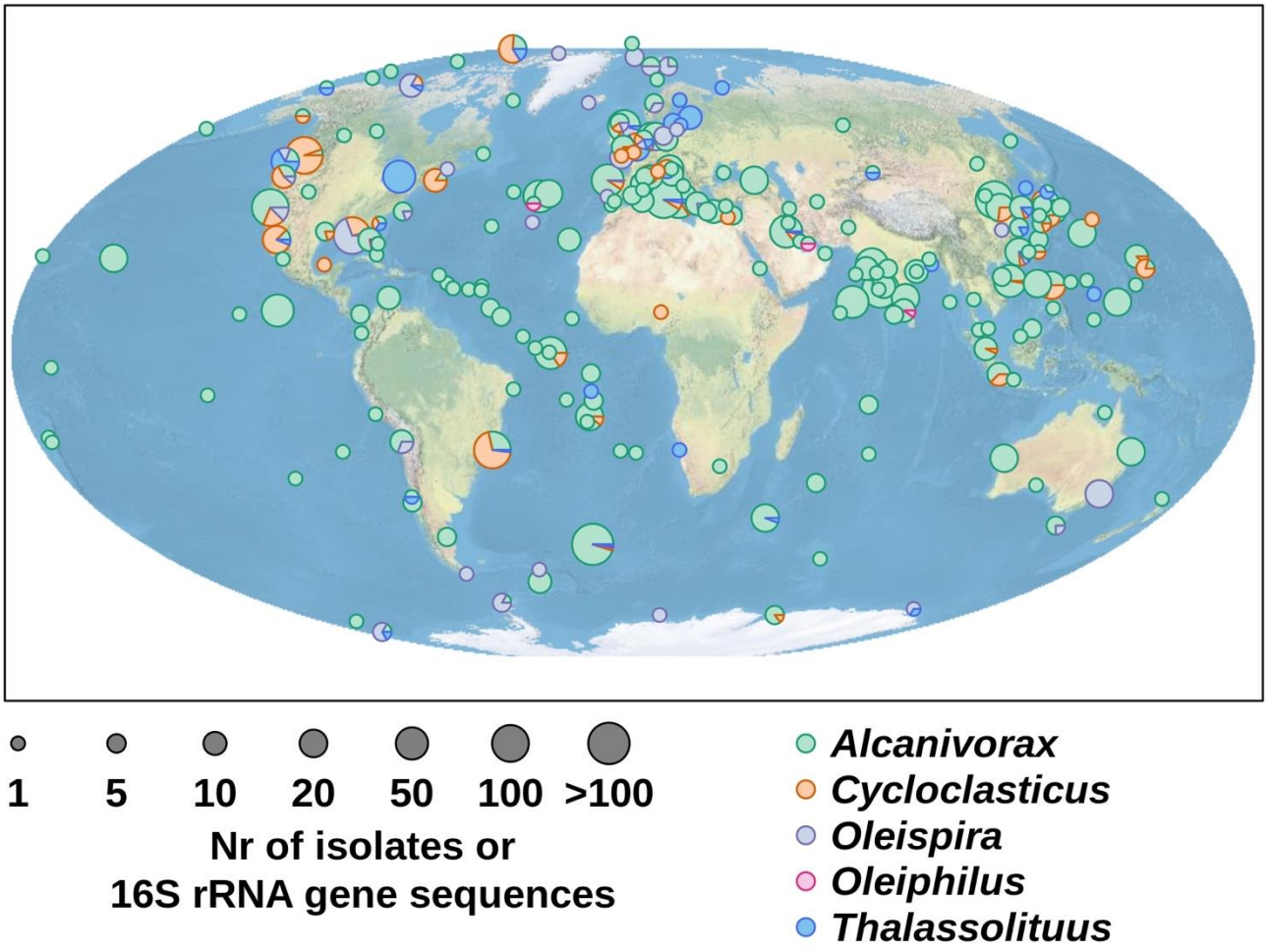


Figure 1 World map showing the distribution of the most prominent and recognized marine OHCB. All available 16S rRNA gene sequences among GeneBank and SILVA databases with tagged coordinates have been used in the figure. When coordinates were not available, the location was aproximately stimated, when it was possible. Figure was developed under R programming environment, using packages *OpenStreetMap* [25].

<<<<<<<<<<<<<<< >>>>>>>>>>>>>>>>>>

As highlighted in recent reviews, there are several biotic as well as abiotic sources from which hydrocarbons, albeit in small quantities, can enter the ocean and sustain the emergence of OHCBs in both pristine and remote areas [13,26]. One of the ubiquitous sources of biotic hydrocarbons is chlorophyll A, the central pigment of photosynthesis, which is an aromatic porphyrin ring with side chain of diterpene hydrocarbon (phytol) and is accounting for 0.3% to 5% of the dry weight of microalgal and cyanobacterial cell [26]. It should be noted that, as it has been known for a long time, many microalgae and especially cyanobacteria are capable of producing significant quantities of various types of hydrocarbons, including long-chain alkanes [27,28••,29•,30,31•], with estimated global flux of de-novo produced hydrocarbons exceeding fossil petroleum inputs into ocean by 100-500-fold [32••,33]. Therefore, it does not seem accidental that representatives of *Alcanivorax*, *Thalassospira*, *Oleibacter* as well as new genera and species of OHCBs (*Polycyclovorans*, *Algiphilus* and *Porticoccus hydrocarbonoclasticus*) have been commonly reported to be associated with many species of phytoplankton (diatoms, dinoflagellates, coccolithophores) [13,26,34-38]. In this regard, the global distribution of OHCBs in the oceans can be explained by their conquest of still poorly studied and underexploited biotopes - the cell surface or phycosphere [26] of marine phytoplankton. The association of some OHCB taxa with these primary producers raises important questions regarding their ecology and their contribution to ocean wellness. Oil pollution and biogenic production of hydrocarbons by phytoplankton are corresponding aspects of the long- and short-term hydrocarbon cycles in the oceans, in which OHCB communities, both phycosphere-associated and free-living in seawater environments, play a significant role.

Marine OHCBs and the plastisphere

In addition to being very important for the sustainability of ocean ecosystems on a planetary scale in respect to the hydrocarbon degradation, OHCB appear to play an additional and yet

underestimated role as an important part of the marine '*plastisphere'*, the newly recognized microbial assemblages colonizing and modifying plastics released in the ocean [39-44]. Petroleum-derived synthetic plastics, including low- and high-density polyethylene (LDPE and HDPE), polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), polyurethane (PUR), and polyethylene terephthalate (PET), are currently major pollutants of marine environments across the globe. It has recently been estimated that between 4.8 and 12.7 million Metric Tons (MTs) of plastic have entered the ocean annually over the past decade, largely due to improper land-based waste management [45]. According to the Plastics-Europe, the global yield of plastics reached 348 million MTs in 2018 [46]. Without major interventions in waste generation and recycling, it was predicted that by 2025, the cumulative amount of mismanaged plastic wastes entering the ocean could reach almost 250 million MTs [45,47]. On the other hand, the production of biodegradable bioplastics (BBPs) has been gradually increasing, as they are considered as promising alternatives to common petrochemistry-based polymers. In 2018, over 2 million MTs of BBPs was produced worldwide with the major BBP types including poly(lactic acid) (PLA, ~25%), poly(butylene succinate) (PBS, ~11%), poly(butylene adipate terephthalate) (PBAT, ~12%), polyhydroxyalkanoates (PHA, ~6%), and starch blends (~44%) [46]. The number of scientific studies focused on BBPs alone has been rapidly increasing with almost 18,000 publications in PubMed as of March 2021. However, BBPs polymers are recalcitrant in the marine environment, as exemplified by Napper and Thompson [48] and represent an emerging group of pollutants and, at the same time, a new substratum for microbial colonization. Recent studies have showed that the current mass of ocean plastic is in the concentration range from between 0.1-1.0 particles m^{-3} in the water column, and from 10^3-10^4 particles m^{-3} in sediments [49] and only 1% plastics entering the marine environment are observed floating at sea [41,50]. This observation is the opposite of the fact that over 65.5% of the

150 world's plastic is represented by lightweight polymers with a lower density than seawater
151 [51].

152 The interest to marine *plastisphere* has recently stimulated numerous studies, which
153 generated a breadth of data [40,52•,53]. Similar to the biofouling that occurs with any inert
154 solid material entering the sea, the plastisphere develops rapidly on the surface of plastic
155 debris when nutrients, carbon sources and energy are available. These determinants, as
156 well as salinity, temperature and solar irradiation, are more likely to influence the
157 development of a particular *plastisphere* [41], which may significantly differ in microbial
158 composition compared to free-living or particle-associated microbial populations in the
159 surrounding water [54. In addition, the type, size and status of the plastic itself (e.g. virgin or
160 weathered) also affects the formation of the *plastisphere*, composition of which is to some
161 extent characteristic for particular type of plastic [55••,56,57].

162 Given that common plastics are derived from fossil fuels, and especially, when oligomers
163 and plastic additives (plasticizers) are available as a source of carbon and energy, one
164 would expect that *plastisphere* should be enriched with OHCBs. The high hydrophobicity of
165 petro-sourced polyolefins (PE, PP and PS) as well as PET, PVC, and PUR creates a strong
166 interface when these materials are immersed in seawater, hindering attachment of the
167 majority of planktonic hydrophilic microbiota. However, these surfaces can be quickly
168 covered by marine microorganisms, possessing hydrophobic outer membrane. Worth to
169 mention, the regulation of the hydrophobicity of outer membranes from neat and hydrophilic
170 to indented and hydrophobic has been documented in a number of studies on marine
171 OHCBs [6,58-61]. Following these expectations, we have attempted to validate the role of
172 OHCBs in light of their abilities to both primary colonization (pioneering) and decomposition,
173 at least partially, different types of marine plastic debris. A more targeted objective was to

elucidate the specific role of OHCBs in the enzymatic (hydrolytic or redox) modification of the original polymer structure.

Marine OHCBs play important role in primo-colonization of plastic.

Although there are many studies on the microbial composition of plastic biofilms, few studies have been devoted to detailed analysis of the successive phases of plastisphere's development [52•,53]. Like biofouling [62,63], this process can be divided into three sequential events: (i) "primo-colonization" phase, which implies the colonization of pioneer bacteria on the plastic surface, which form the first layer of the original biofilm; (ii) a "growth phase" that promotes irreversible attachment through formation of extracellular matrix such as pili, adhesion proteins and exopolymeric substrate matrix (EPS) produced by both primary and secondary species; (iii) the "maturation phase" occurs through diverse, competitive or synergistic interactions between cells with further recruitment or loss of species [56,64]. The role of OHCBs as key colonizers was first demonstrated by investigating the different phases of the colonization of polyolefin-based plastics, namely virgin low-density polyethylene (LDPE), PE with prooxidant (OXO), and artificially aged OXO (AA-OXO) [56]. Succession of plastisphere formed on plastic pieces was monitored for 45 days, with all three developmental phases observed. Notably, OHCBs belonging to genera *Alcanivorax*, *Oleiphilus* and *Thalassolituus* were especially abundant in the plastisphere during the primo-colonization phase and tended to decrease thereafter [56]. It is very likely, that LDPE attracts marine OHCBs independently of its status, since primo-colonization by these organisms was also observed on the weathered LDPE [65]. Similar to the research's data of Dussud et al. [56], the enrichment of a prominent OHCB, *Oleiphilus messinensis*, on both non-weathered and weathered LDPE only occurred at early stages of colonization (i.e., after 2 days of incubation in coastal marine water [Mallorca, Spain]; where they represented 3.7% and 5.8% of relative abundance, respectively, vs. 0.6% on glass controls) [65]. To

some extent, a similar observation was obtained during colonization experiments conducted with virgin high-density polyethylene (HDPE) microbeads (1-4 µm; 0.96 g cm^{-3}) [66]. Incubation of seawater with HDPE for 108 hours stimulated the activity and enrichment of six OTUs, two of which were closely related to OHCBs of the genera *Alcanivorax* and *Oleispira*. The authors hypothesized that, due to an increase in oxygen consumption during incubation with HDPE as compared to control, these specific organisms were uniquely adapted to use organic carbon from virgin HDPE microbeads, likely plastic additives and /or plasticizers, as carbon and energy sources, pointing at their potential for the bioremediation of this type of plastic [66]. The plastisphere succession studied during colonization of three types of polyethylene terephthalate (virgin and weathered PET power [particles <300 µm] and amorphous PET films [250 µm thickness]) for 42 days led to slightly different results [56]. In more details, members of *Piscirickettsiaceae*, the family accomodating the genus *Cycloclasticus*, were found in significant numbers (relative abundance 4.35%) during all phases of plastisphere development on all type of plastic studied. Two different amplified sequence variants (ASV), ASV8 and ASV15 affiliated to *Alcanivoraceae* reached their maximum abundance of 15% and 2.35%, respectively, but at later stages of the plastisphere formation. Notably, while ASV8 was visualized only on virgin PET powder and amorphous PET film, ASV15 was found to grow on all type of plastic studied. The joint participation of *Alcanivorax* and *Cycloclasticus* species in formation of a mature biofilm on the surface of PET films was confirmed in a separated study of Denaro et al. [67]. Interestingly, ATR-FTIR and SEM analyses revealed that most significant alterations (formation of small cracks and cavities) of the surface of PET films was mainly caused by the bacterial consortia enriched on either tetradecane or diesel [67].

Plastic-degrading enzymes from HCB. As indicated above, marine OHCBs are likely to survive in pristine environments through the use of alkanes secreted either by marine

cyanobacteria [30,31•,32••] or other eukaryotic primary producers [69]. Additionally, they appear to have a strong hydrolytic capacity towards aliphatic polyesters, both naturally occurring (e.g. polyhydroxyalkanoates [PHA]) and industrially produced (e.g. polybutylene succinate [PBS], polycaprolactone [PCL], polyethylene succinate [PES] and polylactic acid [PLA]) [70-72•]. Nowadays these aliphatic polyesters, referred above as BBPs, still account for a small fraction of the global polymer market, but fortunately, consumer demand for these "green" alternatives to traditional non-biodegradable petro-based materials is steadily growing [73,74]. Although the molecular mechanisms underlying the ability of OHCB strains to degrade such polyesters are currently unknown, genomes of OHCBs encode a large array of enzyme candidates that potentially can be active on BBPs. Indeed, numerous enzymes from *Alcanivorax, Oleispira*, and *Cycloclasticus* spp., either cloned from their genomes or recovered from metagenomic DNA fragments in crude oil enrichments and attributed to these organisms, were active on polyesters, including PLA, PBS, PHB and PET oligomers [71,72•,75••-79•]. In particular, Tchigvintsev et al. [76•] and Hajighasemi et al. [77] reported three ester hydrolases from the crude oil-seawater enrichments-derived metagenome, two of which were 100% identical to proteins encoded by *A. borkumensis* SK2^{T} genome, one of which, ABO2449, was hydrolyzing poly-(DL-lactic acid) polyesters. Notably, this activity of ABO2449 was found in both emulsified and solid PLA, with the capacity to degrade solid material being of high industrial interest. This polyester hydrolase had a highest activity at 30–37 ° C and retained 32% of the maximum activity at 4 °C, suggesting that its cold-adapted nature [80] (Coscolin et al., 2018). Search for novel and highly active polyesterases from marine metagenomes has led to the discovery of the esterase GEN0105, which hydrolyzes PCL, PLA as well as bis(benzoyloxyethyl)-terephthalate [78•]. Phylogenetic analysis showed that this Family IV polyesterase, was closely related to the alpha/beta hydrolases CUR46463 and WP_055099617 from *Alcanivorax xenomutans*. The recent study on *Alcanivorax* sp. 24 isolated from marine plastic debris, which is able to degrade

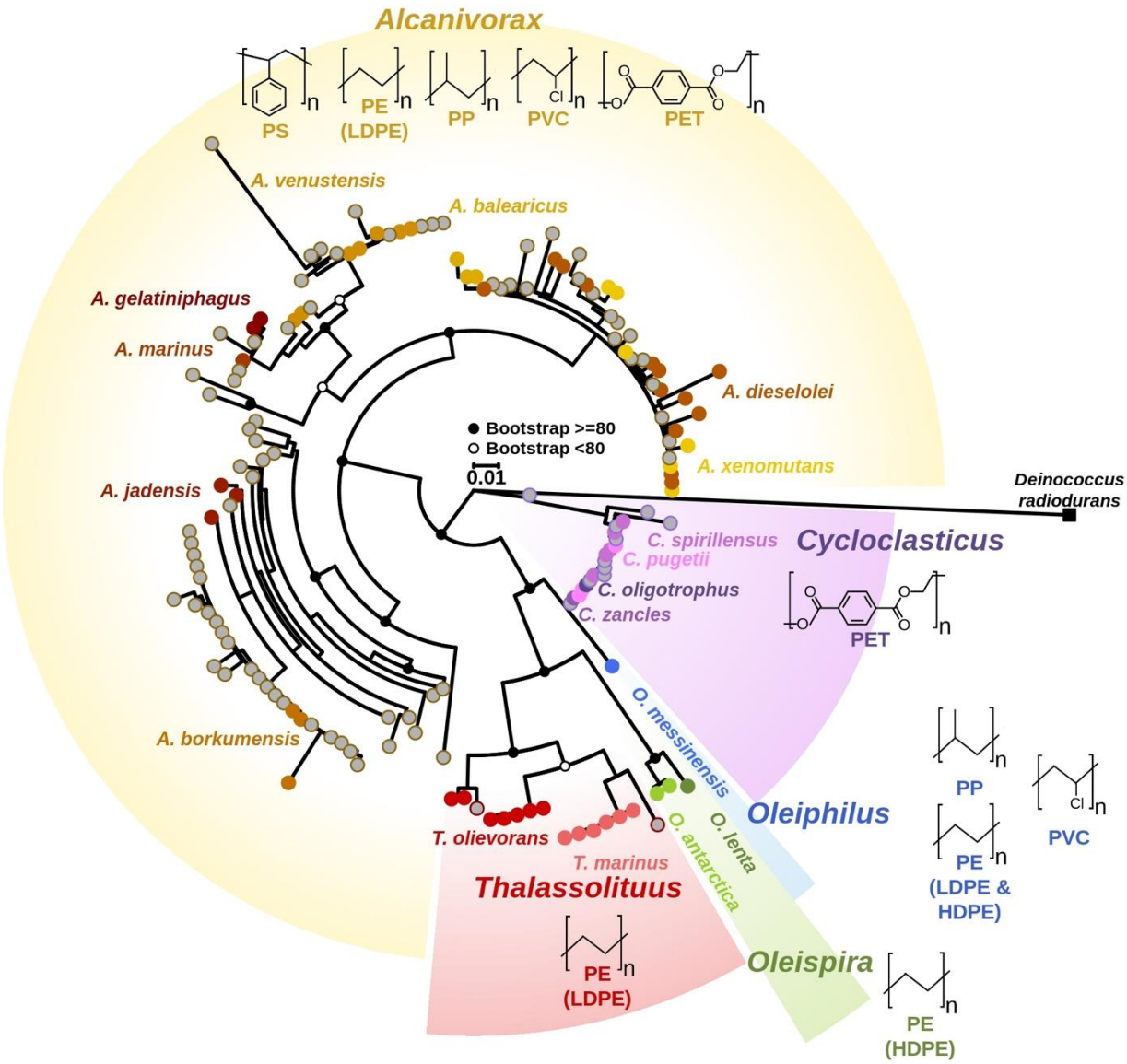
BBP polyesters (PBS, PCL, PES and PHA), has also demonstrated its capacity of bis(2-hydroxyethyl)-terephthalate (BHET) degradation. Further analysis of abundantly secreted ALC24_4107 depolymerase revealed its promiscuous hydrolytic activity towards aliphatic polyesters, which makes it possible to biodegrade both natural and synthetic polyester plastics of anthropogenic origin [72•].

As pointed out elsewhere, many polyesters are of an unnatural origin, exhibit a low bioavailability and are exposed to the microorganisms in their natural environment for a limited period of time, which may be a reason why the evolution of enzymatic pathways for plastic degradation *in situ* is a relatively slow process [41•,81]. Accordingly, the ubiquity of polyester-utilising enzymes, e.g. homologs of PETase from *Idionella* [82••] in marine bacteria and their communities, is correspondingly low, as reflected by the sequencing data analysis [83•,84]. However, it is also known that some families of esterases exhibit an enormous substrate promiscuity and some can utilize, e.g. 74 from 96 natural and synthetic ester substrates tested [85••]. This also suggests that predictions of enzymatic activities from genomic data may overlook enzymes that are readily available in nature and capable of polyester degradation and points at importance of functional enzyme discovery and experimental activity validation, e.g. proteins with domains of unknown function (DUF) or "unknowns".

Conclusion and outlook

The situation with the biodegradation of both natural and synthetic polyesters looks more optimistic compared to the bioconversion of non-hydrolysable polyolefins of petrochemical origin, such as polyethylene, polypropylene and polystyrene, which is hampered by the high redox potential required to cleave carbon-carbon bonds. However, the ability of OHCB to form thick biofilms specifically on LDPE, and break down this petroleum-based plastic has recently been demonstrated [57]. As mentioned above, bacteria of the family *Alcanivoracaceae* are also able to colonize the HDPE microbeads and, probably, use organic carbon from this type of plastic [66] (Figure 2).

278 <<<<<<<<<<<<<<<<Insert Figure 2 here as conclusion>>>>>>>>>>>>>>>> >>>>



279

280 **Figure 2**

281 Silva-based tree of most prominent members of OHCB in marine *plastisphere* possessing the
282 potential to both colonize and degrade plastics.
283 Phylogenetic tree is based on an alignment of 1,303 positions developed using *Mafft* [86] and
284 trimmed using *trimal* [87]. From there, tree was calculated following a GTR model by Maximum
285 likelyhood method with bootstrapping of 1,000 replicates. Calculations and drawing of the figure have
286 been performed using R programming environment [88] using package *ape* [89]. Branches
287 belonging to unspecific species are filled on grey color. In case of evidences for the degradation of
288 plastic polymers, these are shown next to each genus. *Deinococcus radiodurans* (AF289089) has
289 been used as outgroup.

290 <<<<<<<<<<<<<<<<Insert Figure 2 here as conclusion>>>>>>>>>>>>>>>> >>>>

291

Only four types of enzymes (manganese and soybean peroxidases, laccase and alkane hydroxylases [monooxygenases]) have exhibited the PE degradation activities [90]. Since all OHCBs possess a large repertoire of enzymes of the latter type (AlkB, AlmA and P450 monoxygenases), their participation in degradation of low molecular-weight PE in marine environment could be foreseen. It is worth to mention that a recent quantum mechanics study predicting the catalytic mechanism of P450 monooxygenase suggested that oxygenase-induced free radical transitions may cause the cleavage of the carbon-carbon backbone of polyolefins (PE and PS) [87]. While these studies suggest a fundamental possibility that the biodegradation processes of PE or PS, which is at least partially performed by OHCB, are feasible in principle (Figure 2), more effort is needed to characterize biochemical functions of corresponding enzyme candidates to help predicting the plastics biodegradation pathways and enable the engineering of microbial catalysts for plastics biodegradation or recycling [92•].

With the prevalence of plastic marine debris and the continued growth in plastic production, the impact of plastic on marine ecosystems is likely to intensify. Currently, the possibilities of microbial communities developing on the surface of plastic (marine plastisphere) to change the structure and depolymerize both natural and synthetic polyesters are being actively studied. We expect that OHCBs as part of the plastisphere – by accelerating the characterization of their genetic circuits and repertoires – to make a significant contribution to the biodegradation of plastics in the marine ecosystems.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgement

This work was supported by research funds from the Italian Ministry of University and Research (MUR), PRIN 2017, code: 201728ZA49_002 and by the European Commission's Horizon Work Program H2020-FNR-2020 under FUTURENZYME Project Contract (101000327).

CRediT author statement

Michail M. Yakimov: Conceptualization, Methodology, Writing- Original draft preparation. **Rafael Bargiela**: Software, Data curation, Visualization. **Peter N. Golyshin**: Conceptualization, Writing- Reviewing and Editing.

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